ELECTROPHORETIC EVIDENCE AND PLANT SYSTEMATICS

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ABSTRACT

The study of phenotypes and their variation often provides evidence for phylogenetic inferences in plant systematics. Therefore, it is critical that the phenotypes analyzed reflect as directly as possible the underlying genotypes. The equation between phenotype and genotype is simpler and better understood for evidence obtained by electrophoresis of plant enzymes than for most morphological characters. This article discusses the advantages and limitations of electrophoretic evidence to test hypotheses in plant systematics and evolution. It also summarizes the results of a large number of studies which have utilized this evidence. Three general observations from these studies are: (1). Conspecific plant populations are extremely similar genetically as documented by their very high mean genetic identities, 0.95 ± 0.02. This result suggests that one or a few populations often constitute an adequate sample of a species. (2). Congeneric plant species have strikingly reduced mean genetic identities, 0.67 ± 0.07. However, certain pairs of annual plant species have genetic identities similar to those of conspecific populations. In these cases, the species have been shown to be related as progenitor and derivative with the derivative being of recent origin. (3). The amount of genetic variability within plant populations appears closely correlated with their breeding system, with outcrossing populations substantially more variable than inbreeding ones. The article also describes a number of actual and potential applications of electrophoresis in plant systematics.

Evidence obtained by electrophoresis of enzymes has not been widely utilized by plant systematists although it has dominated the research of many of their zoological counterparts and population geneticists (Manwell & Baker, 1970; Lewontin, 1974; Nei, 1975; Ayala, 1976). This has meant that the strengths and weaknesses of such evidence for solving systematic and evolutionary questions in plant biology have not been sufficiently discussed. The present article is designed to facilitate an efficient evaluation, and emphasizes the unique characteristics of electrophoretic evidence, the requirements for its analysis, and actual and potential applications in plant systematics and evolution.

ELECTROPHORETIC EVIDENCE: ADVANTAGES AND LIMITATIONS

The systematist analyzes phenotypes and their variation and often uses this evidence for phylogenetic inference. Such inferences require that observed phenotypes have a specifiable relationship to unobserved genotypes. The equation between phenotype and genotype is simpler and better understood for electrophoretic evidence than it is for evidence obtained from morphological characters or chromatographic comparisons of secondary metabolites. This follows from the colinearity of amino acid sequence and nucleotide sequence as well as the specificity of enzyme catalysis. It also reflects the fact that electrophoretic evidence is used to answer a very different kind of question than has usually been posed by systematists.

Morphological analysis answers a question such as: Are flower petals with

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ANN. MISSOURI BOT. GARD. 64: 161–180.

conspicuously lobed limbs present in taxon *A* and taxon *B*? Chromatographic analysis asks, for example: Is apigenin present in both taxon *A* and taxon *B*? In contrast, electrophoresis answers a question of a different kind: Does glutamate dehydrogenase have the same electrophoretic mobility(ies) in taxon *A* and taxon *B*? Such a question probes the physical properties of particular enzymes or other proteins on the hypothesis that these properties reveal, to a large degree, the record of accumulated mutations that have taken place in the gene specifying the enzyme. And that when a number of enzymes are considered simultaneously, this record can be evaluated with more precision and objectivity than highly complex morphological features.

The primary observed evidence in studies of electrophoretic variation in natural populations is bands of color in a slab of starch or acrylamide gel. These mark the positions reached by different molecular forms of an enzyme (or other protein) which have migrated through the gel under the influence of an electric field. The enzyme variants are separated because they have different electrostatic charges (a function of the relative number of amino acids with positive and negative charges on their surface). Their migration through the gel may also be differentially affected by their size or configuration. Following their physical separation, the enzymes are identified by a staining reaction based on their catalytic activities. The combination of electrophoresis and staining specificity makes it possible to distinguish particular enzymes among hundreds that may be present in a crude tissue extract.

The different molecular forms of an enzyme that catalyze the same reaction are called isozymes if their polypeptide constituents are coded by more than one gene locus (e.g., lactate dehydrogenase, human ADH). They are called allozymes if their polypeptides are specified by different alleles at a single gene locus; the majority of enzymes routinely studied in natural populations have different allozymic forms.

Allozymes are the biochemical consequence of the substitution, deletion, or addition of amino acids in the polypeptides which comprise the enzyme, and they can be distinguished if these changes affect their electrophoretic migration. Since the amino acid sequence of a polypeptide is colinear to the nucleotide sequence of its coding structural gene locus, allozymes result from gene mutation. Thus, an analysis of protein structure using electrophoresis is, to a first approximation, an analysis of a gene. It is precisely this simple relationship between the bands of color on the gel and the nucleotide sequence of genes that makes protein electrophoresis a powerful analytic tool for systematics.

A major advantage of electrophoretic evidence is that colinearity assures that systematic comparisons can be made between products of genes which are homologous (have a common origin), thus, avoiding problems of convergence and functional correlation often prevalent with morphological characters. Another important advantage is that electrophoretic evidence is precise and directly quantifiable in terms of the number and kinds of enzymes studied, permitting the amount of genetic information utilized to be stated exactly. This is seldom possible with morphological or other characters. A third significant advantage is that comparisons are made with enzymes that are generally always present (with the exception of those selectively turned on or off during development) and little influenced by environmental factors. This avoids the frequent situation that occurs with both morphological and chromatographic data in which the absence of a character in one taxon is interpreted as an indication of a less close phylogenetic relationship between it and other taxa that display the character even though independent evidence is lacking regarding the cause of the character's absence. Another advantage is that problems of a priori character weighting do not occur with electrophoretic evidence because all enzymes examined are accorded equal value in similarity matrices or other methods of evaluating divergence.

The theoretical advantages of electrophoretic evidence, to be sure, are offset by certain shortcomings but, fortunately, these are reasonably well defined. The first problem is that a small number of enzymes is sampled and these may not represent enzymes in general since they are most often involved in some aspect of glycolysis, intermediary metabolism, or in the catalysis of certain general types of bonds (esterases, phosphatases, peptidases). Lack of representativeness is probably less serious for systematics, which has not confined itself to characters thought to be representative, than it is for genetic studies which attempt to estimate the total amount and kind of genetic variation in different kinds of organisms. In any event, the enzymes that are examined comprise a sufficiently large category to give meaningful information about many kinds of evolutionary changes.

A problem which is more critical for systematics is that, even for those enzymes examined, the redundancy of the genetic code means that only about 30% of the substitutions of nucleotides are expected to result in the substitution of amino acids that cause changes in electrophoretic mobility (Shaw, 1970). In addition, allozymes that have identical mobilities do not necessarily have identical amino acid sequences. In fact, recent studies utilizing amino acid sequencing (Boyer et al., 1972), heat denaturation (Bernstein et al., 1973; Singh et al., 1974), and variation in gel pore size (Johnson, 1976) suggest that a single mobility class on a gel may sometimes contain more than one enzyme. This requires that more weight be given to evidence of electrophoretic difference than to evidence of similarity. Additional biochemical tests, however, are available to determine whether allozymes with the same mobility have different amino acid sequences. All in all, electrophoretic evidence should be regarded as providing an underestimate of the actual amount of genetic difference between taxa.

Electrophoretic evidence does not include any information on the number of amino acid differences, or mutational steps, that cause differences in enzyme mobilities. A difference in mobility can reflect a single nucleotide substitution or numerous changes in nucleotide sequence. Thus, electrophoresis can demonstrate that two taxa have different allozymes of phosphoglucoisomerase, but it does not provide information about the amount of difference. This is likely to be greater with increasing phylogenetic distance.

Thus, the equation between phenotype and genotype remains acceptable even when the limitations of electrophoretic evidence are considered because they can be specified and the direction of bias is generally known.

ELECTROPHORETIC EVIDENCE: A FRAMEWORK FOR ANALYSIS

When tissue extracts are subjected to electrophoresis in a starch gel, the pattern of enzyme bands (number, spacing, and intensity) is an expression of the particular enzyme system assayed and its mode of inheritance. For some enzymes (usually esterases, phosphatases, and peroxidases), single individuals display complex patterns with as many as 10 to 15 bands because they possess numerous gene loci that code different molecular forms. In contrast, other enzymes are specified by a single structural gene and individuals might display only a single band following electrophoresis. The number of polypeptide subunits of each enzyme and the allelic state of the coding gene (homozygous or heterozygous) also determine the number of enzyme bands displayed. Thus, for an enzyme composed of a single polypeptide, an individual heterozygous at the coding gene displays two allozyme bands, but if the enzyme is dimeric (composed of two polypeptides), three allozymes are displayed, and if it is tetrameric, five are displayed (Fig. 1). In other cases, the polypeptide constituents of an enzyme are coded by different nonallelic genes, for example, alcohol dehydrogenase in maize (Freeling & Schwartz, 1973) and in sunflower (Torres, 1976), producing still additional variants.

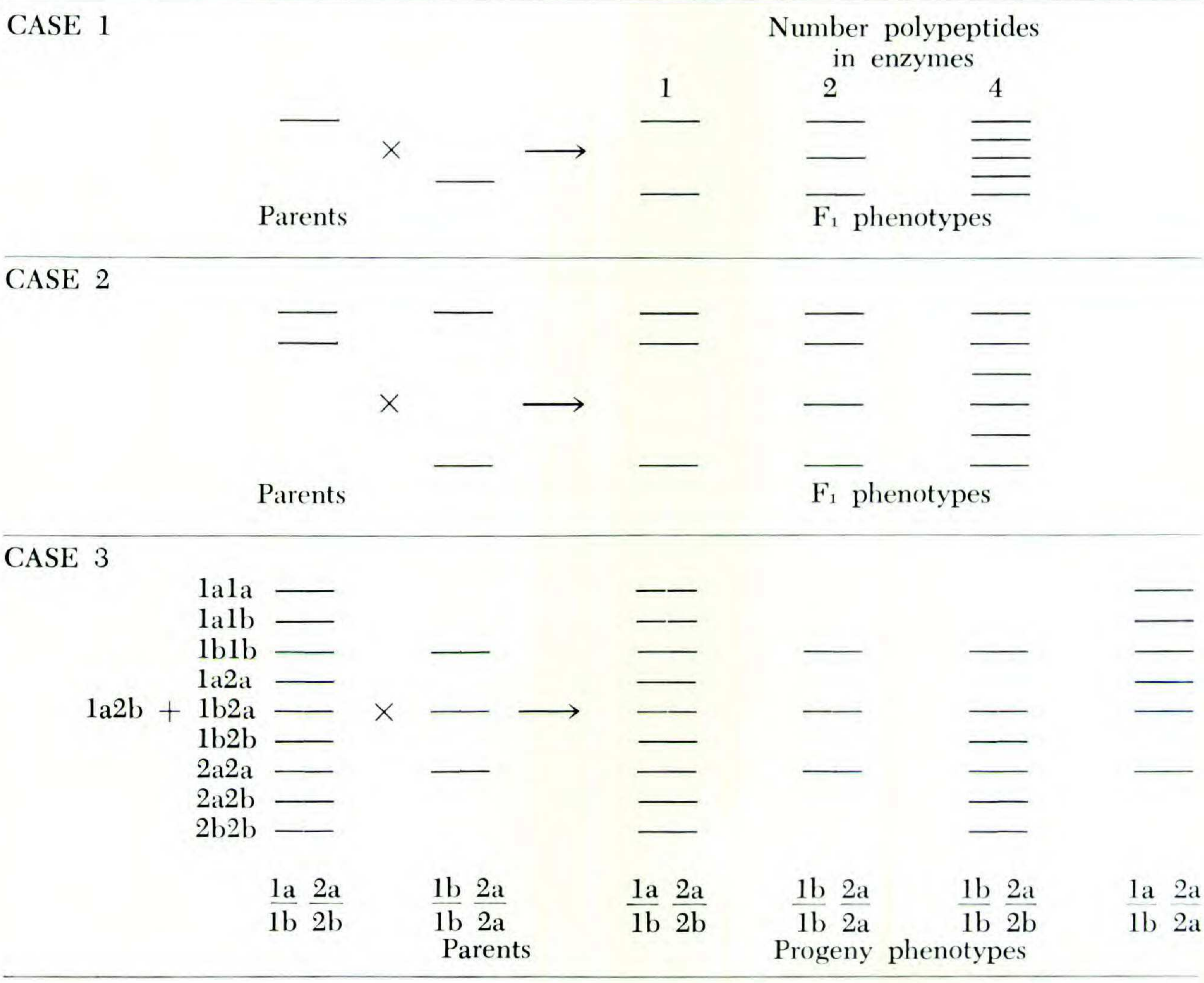
The number of enzyme bands can be reduced if enzymes specified by different genes overlap on the gel because they have similar mobilities, or if an individual is homozygous for a "null" allele (an absence of activity which genetic analysis demonstrates to be allelic to genes that specify active forms of the enzyme). Artifacts that might result from procedures of extraction or electrophoresis can also change band number. In addition to these biochemical factors, the pattern of enzyme bands displayed by different individuals in a population is a function of the amount of genetic variation for the enzyme system.

The presence of so many factors which influence the appearance of the electrophoretic phenotype means that the systematist must reject the temptation to compare electrophoretic data from different taxa by direct inspection, i.e., simply counting the number of bands with similar and dissimilar mobilities. Not only would this approach be biochemically faulty, but it nullifies several of the important advantages of electrophoresis, particularly the inference of enzyme homology, and the precise and quantifiable form of the evidence. The frequent complexity of the electrophoretic phenotype means that, at least for the complex systems that have a number of electrophoretically separable enzyme variants, a genetic analysis is necessary.

Such analysis demonstrates which variant forms of an enzyme system are specified by allelic genes and which by nonallelic genes; i.e., it distinguishes allozymes and isozymes. In addition, in many cases, it rules out the possibility of biochemical artifacts. Genetic analysis also leads directly to a quantitative specification of the electrophoretic data that usually is ordered as follows: the number of structural genes specifying the enzymes examined; the proportion of genes that show variation or, as the geneticist says, are polymorphic in that they have more than a single allele; the number of alleles per gene in the population; and the mean proportion of genes which is heterozygous per individual.

The protocol of formal genetic analysis can often be simplified with electro-

FIGURE 1. These diagrams present three examples of electrophoretic patterns in parents and their hybrids to illustrate that differences between individuals in the number of enzyme bands often do not equal the number of their genetic differences. Case 1 presents a cross between two individuals, homozygous for different alleles at a gene coding an enzyme. The number of bands per individual in their F_1 hybrids depends on the subunit structure of the enzyme. Note that for a dimeric enzyme, a heterozygous individual differs from each of its parents by two bands, but one allele; for a tetrameric enzyme, it differs from them by four bands, but one allele. Case 2 illustrates the same point but uses a cross between individuals homozygous for the same allele at one gene and homozygous for different alleles at a second gene. The F_1 phenotype depends on the subunit structure of the enzyme. In this example, polypeptides specified by the two genes do not have affinity for one another. Case 3 presents a cross between two individuals whose enzyme phenotype is also determined by two gene loci. In this case, the enzymes are considered to be dimeric, and polypeptides specified by both different alleles and different genes associate to form "hybrid" or heteromeric enzymes. The cross is a test cross between a double heterozygote and a double homozygote. If the genes assort independently, the four progeny classes are produced in equal numbers; if they are linked, the parental phenotypes are more frequent than the recombinant ones. Note that the double heterozygote differs from the double homozygote by six bands but only one allele at each gene. The polypeptide structure of each enzyme band is given on the left and the genotype of each individual is given below.



phoretic data because enzyme bands, with few exceptions, show codominant inheritance and segregate as single Mendelian factors (see reviews by Scandalios, 1969, 1974; Jacobs, 1975). This makes it possible to utilize progeny tests to replace formal crosses between individuals with different phenotypes, examine F_1 progeny phenotypes, and analyze phenotypic segregation patterns in the F_2

generation. In the progeny tests, individuals are grown from open-pollinated seeds collected on single plants in nature. Since these individuals all have one parent in common, they necessarily have in common its alleles. For many enzyme systems, study of the segregation pattern of the different variants in such progenies can indicate which of them are specified by allelic genes and which by different gene loci (Brown et al., 1975). However, for very complex systems in which several polymorphic genes and overlaps in the migration of different enzymes are involved, formal analysis is still required. Figure 1 presents examples of such analysis.

Before the introduction of the electrophoretic technique, the study of genetic variation in natural populations was unsatisfactory because it depended on the identification and enumeration of rare recessive mutants that, when homozygous, yielded visible morphological changes. The genetic basis of many of these characters is simple and clearly demonstrable, but they constitute only a very small proportion of the genetic variation in populations. The vast majority of phenotypic characters are apparently controlled by many genes each of which may have different individual effects. These so-called quantitative characters are also often strongly influenced by environmental variation. The result is that the contribution of individual genes of this type cannot be ascertained, and the extent to which they vary one from another in different individuals is undetectable. Thus, one studied either those rare characters controlled by one or two genes with major effects or the much more common characters controlled by many genes that are neither individually identifiable nor distinguishable from environmental influences. In consequence, the traditional methods of studying genetic variation were stymied by the impossibility of equating phenotypes with genotypes.

The electrophoretic procedure avoids most of these problems. In addition, it identifies genes which do not vary on the basis that their enzyme products do not vary in their electrophoretic mobility (within the limits mentioned in the previous section), making it possible to determine the proportion of genes that show variation. This advantage has been considered the "cornerstone" of the method (Hubby & Lewontin, 1966) because previously it was not possible to equate lack of variation with monomorphism at particular genes.

The determination of how many individuals and populations to sample before a confident statement can be made regarding the amount of genetic divergence between taxa is another important consideration in the use of electrophoretic evidence. A definition of the meaning of divergence greatly simplifies this problem. Thus, maximal divergence between two taxa at a gene locus means that they have no alleles in common. Minimal divergence at a gene locus means that the two taxa have similar complements of alleles in similar frequencies. Although the two extremes are connected by a wide variety of intermediate situations, the amount of divergence at a set of genes can often be fitted into a general picture. Thus, if a large number of genes is studied, about 30–50% of them are likely to be monomorphic, another 30–40% will be moderately polymorphic (two or three alleles), and the remaining genes will be highly polymorphic.

The distribution of genes in monomorphic and polymorphic categories permits the systematist to decide, at the outset of a study, the amount of sampling necessary to test a given hypothesis. For example, at a polymorphic gene locus, an allele can be considered common (moderate to high frequency) and widespread, rare (less than 0.05) and widespread, common and local (one or two populations), or rare and local. For certain systematic purposes, one might decide that the first category is most relevant (it is the easiest to sample since alleles here have the highest probability of being included in a sample regardless of strategy). In logistic terms, this means that relatively little effort need be expended to find one more allele which is likely to have low frequency, be local in distribution, or both.

The number of individuals to sample per population is best viewed from the standpoint of how many plants to examine in order to have a 95% certainty of observing all the alleles at a locus which have frequencies greater than 0.05. This problem has been considered by Marshall & Brown (1975) who show that, even in the unlikely case of 20 alleles with frequencies of 0.05 each, a random sample of 120 gametes (60 individuals) will include, with 95% certainty, one copy of each allele. In sum, decisions related to sampling can be neatly bracketed because electrophoretic evidence consists of discrete and precise units of information.

A number of coefficients have been developed to summarize allele frequency data into a single figure that might be used to assess the degree of genetic divergence of taxa (Cavalli-Sforza & Edwards, 1967; Hedrick, 1971; Nei, 1972; Rogers, 1972); however, all of them appear to provide similar estimates (Avise, 1974). The data can also be used to construct dendrograms that cluster taxa according to their similarities (references in Avise, 1974). Another approach, described in the following section, that might be particularly useful for analysis of conspecific populations, makes use of the presence or absence of alleles rather than their frequencies (Gottlieb, 1975).

ELECTROPHORETIC EVIDENCE: APPLICATIONS

Electrophoretic analysis of enzyme variation provides efficient, quantitative estimates of the amount of genic variation within natural populations and the extent of genic divergence among populations. A very large number of electrophoretic studies have been made on animal species. The results appear remarkably consistent: (1) Single populations of both vertebrates and invertebrates contain substantial genetic variability, and perhaps as much as 90% of the total genetic information of their species (review in Powell, 1975; Selander, 1976); (2) Conspecific populations have a very high degree of genic identity (Nei, 1972), often with a mean above 0.90, on a scale of 0 to 1. Their high identity reflects the fact that the same allele is usually fixed at monomorphic genes (as much as 80 to 90% of the genes in vertebrates, for example), and, at the polymorphic genes, only the frequency of alleles differs (Avise, 1974); (3) Closely related species are considerably more differentiated than conspecific populations, with a mean genetic identity around 0.50 to 0.60, which suggests that different species of animals are almost completely distinct in allelic composition at about one-quarter to one-half of their genes (Ayala, 1975). Many of these differences may have evolved since their origins as species because much higher values of genetic identity are observed for pairs of species which apparently originated in the Pleistocene (Nevo et al., 1974; Avise et al., 1975).

In contrast to the wealth of data for animal species, the number of electrophoretic studies of plant species is extremely small (fewer than a dozen groups of congeneric species have been examined for electrophoretic variation in a large number of enzymes) and, consequently, generalizations must still be considered tentative. The paucity of studies with plants is unfortunate because, in many ways, plants are better material than animals for electrophoretic investigations. Thus, they are often easy to grow in high numbers; they need not be killed to obtain a tissue sample so that individuals can be used for additional analysis; progeny-testing to establish the genetic control of enzyme variants is straightforward; natural populations are often spatially and ecologically delimited, permitting coordinated studies of ecological adaptations and amplitudes; phylogenies are often known unambiguously, facilitating analysis of the consequences of speciation; breeding systems are highly variable so that genetic consequences of different amounts of inbreeding can be studied directly and correlated with demographic inputs, etc.

The available electrophoretic studies with plants that are relevant to questions in systematics and evolution can be grouped into four major categories: (1) genetic divergence among conspecific populations; (2) genetic divergence among congeneric species, a subject which has also provided evidence of the genetic and biochemical consequences of speciation; (3) enzyme expression in diploid progenitors and polyploid derivatives; and (4) a heterogeneous group of special-purpose studies (not reviewed here because of space limitations) dealing with themes such as analysis of gene flow across species barriers (interspecific hybridization) (Chu & Oka, 1970; Levin, 1975), consequences of unusual chromosomal pairing mechanisms in *Oenothera* (Levy & Levin, 1975), demographic analysis (Schaal, 1975), the effect of breeding systems on the amount and expression of genetic variability (Allard, 1975; Allard & Kahler, 1971), and genetic diversity and edaphic specialization (Babbel & Selander, 1974).

ELECTROPHORETIC EVIDENCE: CONSPECIFIC POPULATIONS

Electrophoretic variation in enzymes has been examined in natural populations (at least two) of about 28 plant species (Table 1). However, in many respects, the data is very uneven. For example, the number of enzyme systems examined and the number of genes that code them in the different species vary over a five-fold range. In addition, the choice of enzymes is diverse so that in some studies the proportion of genes specifying enzymes that are frequently highly polymorphic (esterases, phosphatases, peroxidases) is high, whereas in other studies many additional enzymes are included that are involved in basic metabolism (such as phosphoglucoisomerase, phosphoglucomutase, glutamate dehydrogenase, malate dehydrogenase, malic enzyme, glutamate oxaloacetate transaminase). Further, the number of populations sampled per species varies widely. The species themselves are nearly all annual but, in other respects, they are heterogeneous including diploids and polyploids, obligate outcrossers and pre-

TABLE 1. Electrophoretic variation for genes specifying enzymes and genetic identities (calculated with the statistic of Nei, 1972) of conspecific plant populations. The data presented are for each species treated as a whole. The species list includes only species in which a large number of enzymes was analyzed in at least two populations.

Species	No. Pop.	No. Enzyme Systems/ No. Genes	% Genes Polymorphic	Mean No. Alleles/ Polym. Gene	Mean Heterozygosity	Genetic Identity	Reference
Avena barbata "I"[a]	9	3/5	0.00	—	0.00	1.00	Clegg & Allard, 1972
A. barbata "II"[a]	7	3/5	1.00	2.00	0.03	0.54	Clegg & Allard, 1972
Clarkia biloba[b]	3	7/8	0.62	3.60	0.15	0.92	Gottlieb, 1974a
C. lingulata[b]	2	7/8	0.62	3.33	0.07	0.90	Gottlieb, 1974a
C. rubicunda[b]	4	8/11	0.64	2.00	0.11	—	Gottlieb, 1973a
Gaura longiflora[c]	3	10/18	0.33	3.16	0.07	0.99	Gottlieb & Pilz, 1976
G. demareei[c]	2	10/18	0.28	3.00	0.05	0.99	Gottlieb & Pilz, 1976
Hymenopappus scabrosaeus[b]	14	6/7	0.71	2.12	0.20	0.97	Babbel & Selander, 1974
H. artemisiaefolius[b]	12	6/7	0.71	2.05	0.21	0.94	Babbel & Selander, 1974
Lupinus subcarnosus[b]	8	7/8	0.88	1.84	0.10	0.97	Babbel & Selander, 1974
L. texensis[b]	10	7/8	0.88	3.12	0.36	0.96	Babbel & Selander, 1974
Lycopersicon cheesmanii[a]	54*	4/14	0.57	2.65	0.01	—	Rick & Fobes, 1975
L. chmielewski[b]	8*	4/14	0.50	3.52	—	—	Rick et al., 1976
L. parviflorum[a]	8*	4/14	0.14	2.00	0.00	—	Rick et al., 1976
Lycopodium lucidulum[a]	16	11/18	0.28	1.39	0.07	0.98	Levin & Crepet, 1973
Oenothera biennis[a]	106*	11/20	0.30	1.40	0.10	0.95	Levy & Levin, 1975
O. parviflora[a]	29*	11/20	0.40	1.55	0.15	0.92	Levy & Levin, 1975
O. strigosa[a]	29*	11/20	0.25	1.30	0.03	0.97	Levy & Levin, 1975
Phlox cuspidata[a]	10	11/16	0.38	2.33	0.01	0.95	Levin, 1975
P. drummondii[c]	10	11/16	0.25	2.25	0.04	0.97	Levin, 1975
Silene maritima[b]	2	13/21	0.29	2.33	~0.14	—	Baker et al., 1975
Stephanomeria exigua subsp. *carotifera*[c]	11	8/14	0.57	4.80	0.09	0.98	Gottlieb, 1975
S. exigua subsp. *macrocarpa*[a]	5	8/14	0.07	2.00	< .01	0.99	Gottlieb, unpublished
S. paniculata[a]	5	14/25	0.04	2.00	0.00	0.99	Gottlieb, unpublished
Tragopogon dubius[a]	6	13/21	0.24	2.50	<0.01	0.97	Roose & Gottlieb, 1976
T. porrifolius[a]	3	13/21	0.10	2.00	<0.01	0.98	Roose & Gottlieb, 1976
T. pratensis[a]	3	13/21	0.00	—	0.00	1.00	Roose & Gottlieb, 1976
T. mirus[a]	6	13/21	0.24	1.20	++**	—	Roose & Gottlieb, 1976
T. miscellus[a]	5	13/21	0.19	2.00	++**	—	Roose & Gottlieb, 1976

[a] Highly self-pollinating.
[b] Self-compatible.
[c] Self-incompatible.
* Strains or accessions.
** Tetraploid species with 40% phenotypic heterozygosity; see text.

dominant selfers, and recently evolved and ancient taxa. However, in spite of these disparities, two results appear well established.

First, the amount of genetic variability within plant populations is closely correlated with their breeding systems (Table 1). Thus, the mean number of alleles per polymorphic gene averages 1.88 ± 0.12 for highly self-pollinating species and 2.86 ± 0.24 for outcrossing ones. The mean proportion of genes that is heterozygous per individual follows suit: 0.032 ± 0.013 for selfers and 0.133 ± 0.026 for outcrossers. These results indicate that breeding system influences not only the degree of genetic homozygosity but also the total amount of variability that can be maintained in plant populations.

Second, conspecific plant populations are extremely similar genetically as demonstrated by their very high mean genetic identity $\bar{I} = 0.95 \pm 0.02$, averaged over all species (Table 1). This is an important result for systematics because it suggests that electrophoretic evidence from one or a few populations very often constitutes an adequate sample of an entire species.

The very high degree of genetic similarity among conspecific populations leads to the suggestion that should populations be discovered which have novel alleles or distinct allele frequencies at more than a few gene loci, such populations are very likely to constitute distinct taxa and should be further examined with this in mind. Such evidence has already been used to identify a subspecies of *Drosophila willistoni* (Ayala, 1973) and a new species of sea cucumber (Manwell & Baker, 1963). It is not unlikely that electrophoretic evidence will also be similarly used to identify hitherto unrecognized plant species.

An approach to the comparison of conspecific populations that considers the representativeness of single populations rather than their identity to one another has also been proposed (Gottlieb, 1975). Designated the Complement Index, it compares the number of nonunique and nonubiquitous alleles (present in more than one but not all populations) in each population with the total number of such alleles identified in all the populations examined. Since the presence of alleles increases the biochemical repertoire of a population, the number of alleles constitutes a very good and easily obtained estimator of the relative potential of different populations for adaptive evolutionary change. The Complement Index would be particularly useful for taxa in which populations contain large numbers of different low frequency alleles, a situation that may be common in outcrossing plants. Thus, 11 populations of *Stephanomeria exigua* subsp. *carotifera* all possessed the same gene at 6 monomorphic loci and 13 high frequency alleles at 8 polymorphic ones, but different numbers of 25 other low frequency alleles (Gottlieb, 1975). Calculation of the Complement Index showed that the populations actually represented subsp. *carotifera* to very different degrees even though they had a mean genetic identity, $\bar{I} = 0.98$ (Gottlieb, 1975). The average population had only about half of all the genes identified in the subspecies as a whole; nevertheless, one of them possessed every one of the nonunique alleles. This population contained more of the genetic resources of subsp. *carotifera* than any other and is the most likely to persist through environmental fluctuations. In addition to identifying such populations (which, with cultivated plants have obvious importance for germ plasm conservation), the Complement

Index could be used to compare the representativeness of populations of different taxa.

ELECTROPHORETIC EVIDENCE: CONGENERIC SPECIES

The large amount of electrophoretic variation in natural populations of plants initially led systematists to presume that extensive surveys within and between populations were required in order to use electrophoretic evidence for meaningful systematic comparisons between species (Turner, 1969). This point of view apparently took hold because genetic studies had only rarely been carried out and therefore it was difficult, if not impossible, to make sense of the complex banding patterns that were observed (many of the early studies unwittingly utilized esterases and peroxidases which are the most difficult systems to interpret). The absence of genetic data and the small number of populations that had been sampled combined to give the impression that the polymorphisms inherent in electrophoretic evidence lessened its value for systematics.

However, now it is realized that once polymorphisms are defined in genetic terms so that "bands" can be equated with alleles and different gene loci, then their presence actually increases the power of electrophoretic evidence for systematic studies since they reveal to a large degree the accumulated record of numerous mutations that have become established in the coding genes. In addition, extensive sampling of conspecific populations is often not necessary for species comparisons because many of the alleles, particularly those with frequencies above 0.20, as well as the genes at monomorphic loci, are now known to be present in most, if not all, populations of a species. However, although the number of populations sampled can be reduced, it remains important to increase the number of enzymes sampled. This would tend to lessen the effect of biases that might result from selecting only enzymes likely to be polymorphic or those limited to any particular biochemical category.

About a dozen studies have been made that provide evidence of the extent of genetic divergence between congeneric species (Table 2). These studies show that most pairs of species have strikingly reduced genetic identities; $\bar{I} = 0.67 \pm 0.07$, averaged over all pairs of species examined. A number of species pairs, however, have very high genetic identities, within the range of those characteristic of conspecific populations. For three of these cases, *Stephanomeria exigua* subsp. *coronaria* and "Malheurensis" (Gottlieb, 1973b, 1976), *Clarkia biloba* and *C. lingulata* (Gottlieb, 1974a), and *Gaura longiflora* and *G. demareei* (Gottlieb & Pilz, 1976), the species are known to be related as progenitor and derivative, respectively, with the derivative being of relatively recent origin.

The three cases represent the three possible pathways of diploid speciation in annual plants, defined in terms of the breeding systems: self-incompatible to self-compatible, self-compatible to self-compatible, and self-incompatible to self-incompatible, respectively. A fourth example of very high genetic similarity between progenitor and derivative diploid species has recently been identified in *Lycopersicon* (Rick et al., 1976). The lack of genetic divergence between the members of each species pair indicates that, shortly after their origin, annual plants, regardless of their breeding system, are still limited genetic versions of

TABLE 2. Nei's (1972) mean genetic identity, $\bar{I}$, between pairs of populations of congeneric plant species.

Species	$\bar{I}$	Reference
Clarkia biloba × *C. lingulata*	0.88	Gottlieb, 1974a
Clarkia rubicunda × *C. franciscana*	0.28	Gottlieb, 1973a
Gaura longiflora × *G. demareei*	0.99	Gottlieb & Pilz, 1976
Hymenopappus scabrosaeus × *H. artemisiaefolius*	0.90	Babbel & Selander, 1974
Lupinus texensis × *L. subcarnosus*	0.35	Babbel & Selander, 1974
Oenothera strigosa × *O. biennis*	0.97	Levy & Levin, 1975
Oenothera strigosa × *O. parviflora*	0.54	Levy & Levin, 1975
Oenothera biennis × *O. parviflora*	0.55	Levy & Levin, 1975
Phlox drummondii × *P. cuspidata*	0.67	Levin, 1975
Stephanomeria exigua subsp. *coronaria* × "Malheurensis"	0.94	Gottlieb, 1973b, 1976
Tragopogon dubius × *T. porrifolius*	0.50	Roose & Gottlieb, 1976
Tragopogon dubius × *T. pratensis*	0.62	Roose & Gottlieb, 1976
Tragopogon porrifolius × *T. pratensis*	0.53	Roose & Gottlieb, 1976

their progenitors and possess very few or no unique alleles insofar as their genomes have been assayed. The species are extracted from the parental repertoire of phenotypic variation and genetic polymorphisms. Thus, the speciation process does not seem to involve early reconstitution of the genome of the derivative species, even though it may possess certain unique morphological traits or other features (Gottlieb, 1976).

The other two examples of high genetic identity between species are not inconsistent with this thinking. Thus, the similarity of *Oenothera strigosa* and *O. biennis* presumably reflects the fact that they have one genome in common (Levy & Levin, 1975). And the high identity of the two species of *Hymenopappus* is concordant with their very close phylogenetic relationship as judged by their high overall morphological similarity; they were maintained as species because they apparently do not hybridize despite extensive parapatric contact (Turner, 1956). However, it is not clear if the results with annual plants will also characterize perennial plant species. This is because perennials, especially long-lived ones, appear to evolve gradually, rather than rapidly and abruptly, and they are more likely to be reproductively isolated by ecological and pollination factors rather than hybrid sterility resulting from chromosomal restructuring.

Electrophoretic evidence has also been used to show that species which appear similar may actually not be so. Thus, *Clarkia franciscana*, a highly self-pollinating species thought to have evolved by rapid reorganization of chromosomes from the morphologically similar *C. rubicunda* (Lewis & Raven, 1958), is totally divergent from that species in a high proportion of its genes (those coding six of the eight enzyme systems assayed) (Gottlieb, 1973a). In addition, *C. franciscana* has a duplicated gene for alcohol dehydrogenase which further distinguishes it from *C. rubicunda* (Gottlieb, 1974b). Such marked genetic differentiation requires that the phylogenetic separation of the two species occurred much longer ago than had been presumed, and makes its proposed mode of origin quite uncertain. Therefore, a reasonable criterion to apply in cases like this is that a species not be accepted as having originated recently from another extant species if it is not electrophoretically highly similar to its putative parent (Gottlieb, 1973a). This criterion has now been satisfied in the four examples described above.

When additional electrophoretic studies are reported, it may very well turn out that such evidence reflects species divergence more sensitively than other types of biochemical analysis. Thus, two-dimensional chromatography of certain flavonoids in *Tragopogon dubius*, *T. porrifolius*, and *T. pratensis*, failed to distinguish a single component that was species-specific (Brehm & Ownbey, 1965), even though the morphological differences between these species are "broad, sharp and absolute" (Ownbey, 1950). But, electrophoretic analysis of many enzymes in North American populations of the three Tragopogons revealed very clearly that they were fixed for different alleles at about 40% of the 21 genes examined (Roose & Gottlieb, 1976). Other groups of plants have not yet been studied so extensively both for electrophoretic variation in enzymes and chromatographic variation in flavonoids and, therefore, it is not possible to know if such a result will prove general. However, this is not implausible since changes in the amino acid sequences of a large number of polypeptides which affect electrophoretic mobilities of enzymes are more likely to reflect early stages of genetic divergence than are changes in secondary metabolites such as flavonoids which are products of enzyme-catalyzed biosyntheses.

Electrophoretic evidence is also likely to be useful in other systematic investigations which require knowledge of the extent of genetic similarity of closely related diploid species. An attractive use will be to examine cases in which one species appears to be a stabilized derivative of hybridization between two other extant species such as *Lasthenia burkei* (Ornduff, 1976), *Potentilla glandulosa* subsp. *hansenii* (Clausen et al., 1940), *Achillea rosea-alba* (Ehrendorfer, 1959), and *Delphinium gypsophilum* (Lewis & Epling, 1959). Another use will be to answer a novel systematic question having to do with the relative similarity of species in different genera. A sample question might be: Are species of *Clarkia* more similar to one another than species of *Baptisia*? Once again the question becomes plausible because of enzyme homology and because electrophoretic evidence is composed of discrete, quantifiable units of information. The comparison of relative taxonomic distance in different genera might eventually lead to the development of procedures to standardize certain taxonomic decisions.

A further application of electrophoretic evidence above the species level takes advantage of its ability to distinguish species with different numbers of genes specifying the same enzyme system. In cases where an enzyme is composed of several polypeptides, the formation of "hybrid" enzymes by the association of subunits coded by different gene loci provides strong evidence for their homology and the origin of one of the genes through duplication. Clearcut examples of such gene duplication have been documented for animal lactate dehydrogenase (reviewed in Markert et al., 1975), hemoglobins (Ingram, 1961), and phosphoglucoisomerase (Avise & Kitto, 1973). Gene duplication is very probably a unique event in the evolutionary history of organisms and, therefore, it can provide evidence of the monophyletic origin of large groups of species and genera.

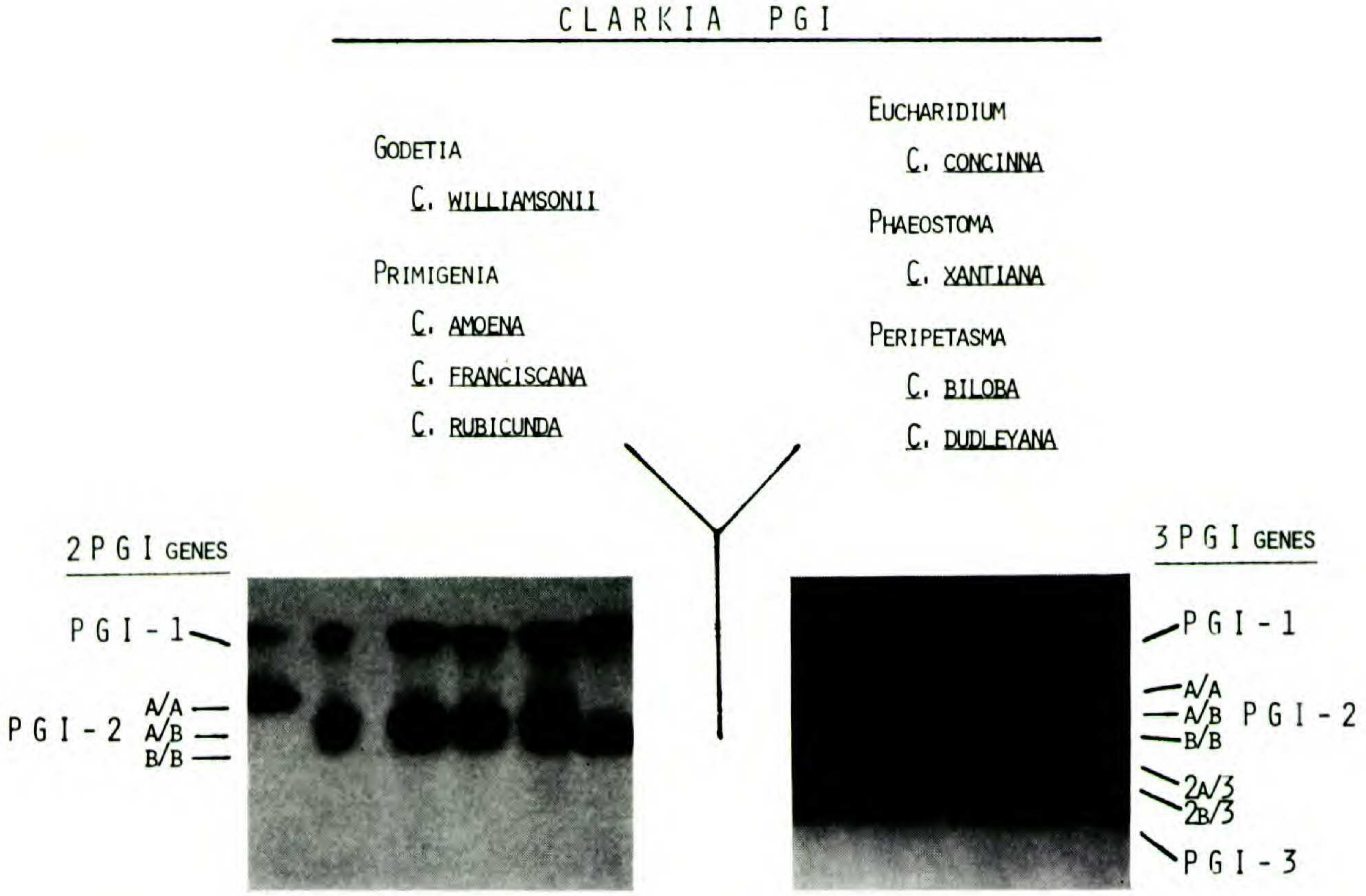

CLARKIA PGI
GODETIA
C. WILLIAMSONII
PRIMIGENIA
C. AMOENA
C. FRANCISCANA
C. RUBICUNDA
EUCHARIDIUM
C. CONCINNA
PHAEOSTOMA
C. XANTIANA
PERIPETASMA
C. BILOBA
C. DUDLEYANA
2 PGI GENES
PGI-1
PGI-2
A/A
A/B
B/B
3 PGI GENES
PGI-1
A/A
A/B
B/B
PGI-2
2A/3
2B/3
PGI-3

FIG. 2. Eight diploid species in five of the seven diploid sections of *Clarkia* which have been examined to date can be divided into two groups on the basis of the number of genes they have specifying phosphoglucoisomerase subunits. The photographs show typical electrophoretic phenotypes for this enzyme system in the two groups. Details of the genetic analysis are described by Gottlieb (1977).

During studies of the genetic divergence of diploid species of *Clarkia*, I uncovered two cases of apparent gene duplication (Gottlieb, 1974b, 1977). The one involving duplicated phosphoglucoisomerase (PGI) is particularly relevant to systematic studies because it tests directly the taxonomic delimitation of sections within the genus proposed by Lewis & Lewis (1955). Genetic analysis of PGI in *Clarkia* has shown that species either have two or three genes specifying these enzymes (Gottlieb, 1976, 1977). To date, eight species in five of the seven diploid sections of the genus have been examined: *Clarkia rubicunda*, *C. amoena*, and *C. franciscana* in the presumed primitive section *Primigenia* have two genes (Gottlieb, 1973a) as does *C. williamsonii* in the *Godetia* section (Price, 1975). Section *Phaeostoma* is represented by *C. xantiana*, *Peripetasma* by *C. biloba* and *C. dudleyana*, and *Eucharidium* by *C. concinna*, and all of these species have three PGI genes (Gottlieb, 1977) (Fig. 2).

The duplication was originally recognized because individuals from species with three PGI genes display more enzymes upon electrophoresis than those from species with two PGI genes. The actual number of enzyme bands observed depends on the allelic state of the coding genes and the affinity of polypeptides specified by the different alleles and the different gene loci. PGI is composed of two polypeptides so that an individual heterozygous for different alleles at a single coding locus normally produces three enzymes by two-by-two association of the two polypeptides (aa, bb, ab). In the Clarkias examined, the gene coding the most anodal PGI appears to be invariant since individuals with either two or three PGI genes have always possessed a single fast PGI; polypeptides specified by this gene, called *PGI-1*, do not form a "hybrid" enzyme with those specified by either of the other genes (Fig. 2).

Thus, the maximum number of PGI enzymes observed in the two-gene species was four (the single fast PGI coded by *PGI-1* plus three enzymes in individuals heterozygous at *PGI-2*). However, as many as ten enzymes have been observed in the three-gene species because polypeptides specified by the duplicated gene, called *PGI-3*, from "hybrid" enzymes with those specified by the original *PGI-2* gene. Thus, when both *PGI-2* and *PGI-3* are heterozygous, four different polypeptides are made which aggregate to form nine distinguishable enzymes (Fig. 1, case 3), and *PGI-1* codes a tenth enzyme band.

The duplication is thought to have originated by the generation of a duplicated chromosome segment in a progeny of a cross between individuals differing for chromosomal rearrangements, possibly a partially overlapping reciprocal translocation (reviewed in Burnham, 1962). Self-fertilization would make the segment homozygous in a few generations. This mode of duplication is likely in *Clarkia* because in this genus, species are self-compatible and differ by large numbers of reciprocal translocations. Such duplications will not be linked; recent genetic analysis (Gottlieb, 1977) in *Clarkia xantiana* has shown that *PGI-2* and *PGI-3* assort independently, which is consistent with the proposed duplication process.

The species with three PGI genes can be considered a monophyletic group that traces back to an ancestor that branched away from the *Primigenia-Godetia* stock. That two genes is the ancestral number is directly supported by the observation that *Oenothera*, the most closely related genus to *Clarkia*, also has two genes for PGI (Levy et al., 1975) as does *Gaura* (Gottlieb & Pilz, 1976), another genus in the same tribe of the Onagraceae. The utilization of the number of genes coding specific enzymes to classify groups of species into monophyletic assemblages appears not to have a parallel in current systematic research. Gene duplication provides a strict homology, absent with most morphological characters, because convergence in particular structural genes is highly improbable since it would require a very high number of mutational changes to alter the coding properties of a different nonhomologous locus.

ELECTROPHORETIC EVIDENCE: POLYPLOID SPECIES

The ancestry of most allotetraploid species (tetraploids are used as an example of polyploids) can, in principle, be traced back to a chromosome doubling in a diploid individual which was produced by hybridization between differentially adapted populations. The initial allelic composition of the tetraploid plants is a direct function of the degree of genetic divergence of the diploid progenitor populations and is likely to be substantially greater if these represent species. This follows because species have a very much higher probability than conspecific populations of possessing different alleles at their monomorphic gene loci and nonoverlapping complements of alleles at polymorphic genes. After the events of its origin, the courses of evolution in the tetraploid and its diploid parents are independent which suggests that the more ancient the tetraploid, the less likely will it retain the alleles it inherited in an unmutated state, and, likewise, alleles will continue to evolve in the diploids. Thus, the ability to identify the diploid parents of a tetraploid species with electrophoretic evidence depends on numerous factors having to do with the amount of divergence of the diploids at the time of tetraploid origin as well as subsequent evolutionary events.

It follows that evidence brought to bear on the phylogeny of a tetraploid species not be limited to a few enzymes or to a limited class of proteins, and that particular attention be paid to specific protein homology and the mode of inheritance of the proteins examined in order that an absence or difference in mobility can be interpreted in terms of genetic changes. This prescription has been ignored in numerous studies of diploid and tetraploid plant species which have employed only one or two enzyme systems or have sampled only seed proteins and, consequently, many of these studies have uncertain value and are not dealt with here.

In general, electrophoretic analysis has demonstrated that polyploid species express, additively, enzymes present separately in their diploid parents. This result has been reported, for example, in wheat (Hart, 1969; Mitra & Bhatia, 1971; Barber, 1970), cotton (Cherry et al., 1972), *Nicotiana* (Smith et al., 1970; Reddy & Garber, 1971; Sheen, 1972), *Phaseolus* (Garber, 1974), *Stephanomeria* (Gottlieb, 1973c), and *Tragopogon* (Roose & Gottlieb, 1976). If the duplicated genes of polyploids specify different polypeptide subunits of multimeric enzymes (those that are composed of more than one polypeptide), additional "hybrid" enzymes are produced which are not expressed in a diploid parent if it lacks both coding alleles. For many enzymes, the polyploid species is a "fixed heterozygote" because all of its individuals express a multiple enzyme phenotype that reflects their possession of different coding alleles inherited from the diploid species. This multiple enzyme phenotype does not exhibit genetic segregation because, at meiosis, chromosome homologues often pair preferentially so that genes inherited from both diploid parents go to the same pole and each gamete receives one copy of each of them. At fertilization, each gene is made homozygous, but their presence in duplicate means that a heterozygous (multi-enzyme) phenotype can be produced in the tetraploid. The multiplicity of enzymes in a polyploid species may extend the range of environments in which normal development can take place, and this is a reasonable hypothesis to account for the frequent wider distribution of tetraploid species relative to the diploids in many genera (Barber, 1970; Manwell & Baker, 1970; Gottlieb, 1976).

The general observation that the enzymes usually assessed by electrophoresis are expressed additively in polyploid species (the only apparent exception is a study in wheat, Sing & Brewer, 1969) may reflect, to some extent, the relatively recent origin of the tetraploids which have been examined. Although ancient polyploid complexes have not yet been studied by electrophoresis, many recently evolved polyploids have considerable systematic and evolutionary significance because they provide critical evidence regarding the initial genetic and biochemical consequences of this type of genome doubling.

The most extensive comparison of enzyme variation in diploid and tetraploid species has been made in *Tragopogon* (Roose & Gottlieb, 1976). The three diploid species, *T. dubius*, *T. porrifolius*, and *T. pratensis*, were introduced to America from Europe during recent times. They are sharply delimited morphologically without overlap in a number of characters (Ownbey, 1950). In southeastern Washington and adjacent Idaho, Ownbey (1950) discovered that interspecific hybridization between them had given rise to two different tetraploid species: *T. dubius* and *T. porrifolius* were the parents of *T. mirus*, and *T. dubius* and *T. pratensis* were the parents of *T. miscellus*. These two tetraploid species represent the only unambiguous examples of the very recent natural origin of polyploid species.

Electrophoretic evidence revealed that the three diploid species in North America are completely divergent (monomorphic for different alleles) at about 40% of the 21 genes that were examined, a result fully concordant with their morphological differentiation. The tetraploids inherited both alleles at each of these genes: *T. mirus* expresses an additive pattern for nine genes and *T. miscellus* for seven genes, including five in common with *T. mirus*. In both tetraploids, the additive pattern includes novel hybrid enzymes not produced in the diploid species. Each of the enzyme phenotypes in the tetraploids was fully accounted for by simple additivity of the polypeptides specified by genes inherited from its respective diploid parents. The observed patterns fully confirmed the ancestry of both tetraploids which was proposed by Ownbey (1950). These results, as well as others, clearly indicate that electrophoretic analysis of large numbers of enzymes can be an extremely useful and precise probe to identify diploid progenitors of polyploid species.

In summary, electrophoretic evidence can be used to test many different types of hypotheses regarding genetic divergence that have already been generated in plant systematics. In addition, its unique perspective will probably help to link questions previously considered to require only systematic or evolutionary evidence to different sources of evidence in biochemistry and plant development.

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